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WESTERN ANALYSIS OF ARABIDOPSIS CELLULOSE SYNTHASE EXPRESSION

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ABSTRACT

Cellulose in vascular plants is synthesized by the cellulose synthesis complex (CSC) located in the cell membrane. The *Arabidopsis* CSC is composed of ten cellulose synthase (CesA) protein isoforms, which, although highly similar, have specialized in the ability to produce the various cell wall types required to make a plant. Primary and secondary cell wall synthesis each require three unique, non-redundant CesA isoforms for CSC assembly and function. CesAs 1, 3, and 6, are required for primary cell wall synthesis, while CesAs 4, 7, and 8 form the CSC for secondary wall cellulose biosynthesis. Our lab has recently answered the long-standing question of CesA stoichiometry within the CSC, leading to the most current CSC model consisting of a hexamer of CesA trimers in a 1:1:1 ratio. This work aims to contribute to the understanding of CesA stoichiometry. The work described herein was integral to the elucidation of an equimolar CesA stoichiometry. I performed quantitative analysis of CesA protein levels along the *Arabidopsis* stem, which represent a spectrum of development. These results suggest that the elucidated 1:1:1 stoichiometry is fixed throughout the progression from primary to secondary cell wall synthesis. Isoform stoichiometry determination gives a greater understanding of how and when plants produce different CesA proteins and offer insight into the process of cellulose synthesis. This finding was crucial for the interpretation and validation of Tien lab's determination of an equimolar stoichiometry within a single sample. Additionally, this work aids in mapping CesA protein expression to provide a greater understanding of cellulose synthesis so that one day this process can be manipulated to increase energy yield.

TABLE OF CONTENTS

LIST OF FIGURES iii

LIST OF TABLES iv

ACKNOWLEDGEMENTS v

Chapter 1 Introduction 1

Chapter 2 Results 6

2.1 Purification and Specificity of CesA Antibodies 6
2.2 Quantification of CesA Protein Levels in the Arabidopsis Stem 7

Chapter 3 Discussion 11

3.1 Synthetic Peptides are Capable of Raising Antibodies specific to CesA isoforms 11
3.2 CesA Stoichiometry is Fixed Throughout Stem Development 12

Chapter 4 Materials and Methods 16

4.1 Plants and Growth Conditions 16
4.2 Affinity Purification of Peptide Antibodies 16
4.3 Plant Stem Preparation and Phenol Protein Extraction 17
4.4 Western Blotting and Quantification 18

BIBLIOGRAPHY 19

LIST OF FIGURES

Figure 1. Purification and specificity of CesA antibodies by immunoblot. 7

Figure 2. Arabidopsis model and sectioning scheme. 8

Figure 3. Immunoblot Analysis of *Arabidopsis* PCW CesA Expression.....9

Figure 4. Immunoblot Analysis of *Arabidopsis* SCW CesA Expression.....10

LIST OF TABLES

Table 1. Peptides used to generate CesA antibodies.....6

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Chapter 1

Introduction

Cellulose synthesis is an important subject in current energy sustainability/ alternative energy research. As a vital structural component of plant cell walls, it is the most abundant organic polymer on Earth. There is a staggering amount of cellulose byproduct in agricultural, manufacturing, and other industries involving plant matter. This leftover cellulose can potentially be harnessed as biofuel instead of simply being left to break down. Biofuels are already made from energy crops like corn and sugar cane, but these are important food crops and widespread use may affect availability to people. One way of increasing energy yield or efficiency is by genetically modifying plants to make biofuel synthesis or combustion more efficient. But to further apply this idea, we must understand how cellulose is made by plant and how we can affect that process to increase cellulose yield, more efficiently make cellulose, etc. Mapping CesA expression will provide a greater understanding of cellulose synthesis so that one day this process can be manipulated to increase energy yield.

Cellulose is a vital structural component of plant cell walls as well as algae and bacteria, providing mechanical support as well as supporting roles in cell growth and protection (Kovalenko, 2010). It is a linear polymer of β-1, 4-D-glucose, where adjacent glucose rings are rotated 180° relative to one another, such that the repeating unit is cellobiose. A variable number of parallel glucan strands are synthesized by a cellulose synthesis complex to form a crystalline cellulose microfibril (Kovalenko, 2010). The microfibril is notable for its biophysical properties, chief being its role as the load-bearing component of the cell wall (Kovalenko, 2010). Cellulose

microfibrils are in turn cross-linked by hemicelluloses – a family of amorphous, branched heteropolymers with a backbone composed of β-1, 4-glucose residues like cellulose (Kovalenko, 2010). In terms of conversion, hemicelluloses are relatively weak compared to cellulose, which is strong, crystalline, and resistant to hydrolysis. Lignin, which 'space fills' and lends rigidity to the cell wall, and pectin, which allows for cell growth, are also noteworthy components of the dicot cell wall matrix (Kovalenko, 2010). In the secondary cell wall, cellulose can be organized through the formation of macrofibrils, or bundles of multiple cellulose microfibrils (Kovalenko, 2010). A major contribution to the strength of cell walls is the extensive hydrogen bonding network made possible by the several free hydroxyl groups present of every monomeric unit. This allows for a staggering number of hydrogen bonds, both inter- and inra- molecular, providing the strength to support of the Earth's largest organic structures, the giant sequoias.

Vascular plants possess both thin primary and thicker secondary cell walls. The primary cell wall is synthesized during growth, while the cell is still enlarging (Cosgrove et al., 2012). It is highly hydrated, and contains only 15-40% cellulose by dry weight. It is a relatively thin and flexible structure, composed of cellulose and matrix polysaccharides in a conformation that allows for expansion and remodeling (Cosgrove et al., 2012). The structure gives rise to properties desirable for plant growth: tensile strength for support, extensibility, and the ability to incorporate additional structures in the growing plant. The secondary cell wall, in contrast, provides strength and rigidity to plant tissue that has stopped growing: it is strong, able to resist compressive and tensile pressure, but no longer extensible (Cosgrove, 2005). Secondary cell wall material is deposited onto the primary wall matrix after cell expansion has stopped, and is much less hydrated than the primary cell wall (Cosgrove et al., 2012). Relative to the primary cell wall,

it has a higher percentage of uniformly oriented cellulose strands and is highly lignified, making it ideal for plant support as well as water conduction in the xylem (Cosgrove et al., 2012).

The cellulose biosynthesis machinery was first observed as granules on the cell plasma membrane by electron microscopy in 1972 (Robinson, 1972). These granules were later found to be enzymes linked together to form a plasma membrane complex associated with the ends of cellulose microfibrils, and thus dubbed terminal complexes (Brown et al., 1976). Analysis by freeze-fracture microscopy showed that these terminal complexes formed intramembrane rosette structures (Mueller et al., 1980). The cellulose synthesis machinery, now called the cellulose synthesis complex (CSC), was revealed to be six-particle rosette structure about 25-30 nm in diameter. The identification of the first plant cellulose synthase (CesA) and immunogold labeling of the CSC showed that CesA proteins were the catalytic subunits of the rosette CSC (Pear et al., 1996; Kimura et al., 1999). Cellulose synthases belong to a class of glycosyltransferases – enzymes that form glycosidic linkages. Specifically, CesA catalyzes the addition of glucose to a growing homo-polymer of β-1, 4-D-glucose from the substrate, UDP-glucose. Despite the rotated conformation of each glucose residue relative to the previous residue, only one CesA active site is required to catalyze cellulose chain lengthening (Morgan et al., 2013).

The plant CesA, first characterized in rice and cotton, is homologous to the bacterial BcsA genes, but shares weak sequence homology (Saxena et al., 1990; Pear et al., 1996). In plants, there are two transmembrane helices (TMHs) toward the N-terminus, and six more close to the C-terminus that may serve to anchor the protein into the membrane, in contrast to the 4 + 4 TMH organization of BcsA (Slabaugh et al., 2014). In between the two TMH groups lies the central cytoplasmic region containing the glycosyltransferase (GT) domain. Within the GT domain, plant CesAs contain two regions not present in the bacterial cellulose synthase, BcsA.

The first is highly conserved among all plant CesAs and is therefore referred to as the plant-specific conserved region (P-CR). The second is a variable region containing low sequence similarity between CesAs within a species, which has been classified as the class specific region (CSR). Further analysis reveals that the CSR is one of five regions in plant CesA that determine class specificity (Buckeridge et al., 1999; Kumar et al., 2015).

The number of CesA isoforms in plants varies by species; *Arabidopsis thaliana* contains ten CesA genes (Richmond, 2000). CesAs 1, 3, and 6 are required for primary cell wall synthesis, whereas 4, 7, and 8 are required for secondary cell wall formation (Taylor et al., 2003; Persson et al., 2007; Timmers et al., 2009). All three proteins are non-redundant and necessary to assemble into a functional CSC. Within the model of the SCW CSC, if one subunit is absent, the remaining two will not assemble, indicating that there is some interaction between the three subunits that is necessary for subunit assembly and/or stability. All three subunits are colocalized in the plasma membrane, suggesting that complexes are formed in the endoplasmic reticulum and transported to the plasma membrane (Gardiner et al., 2003). CesAs 2, 5, and 9 are tissue specific and are partially redundant with CesA 6. CesA 10 serves a minor or unknown role (Desprez et al., 2007; Persson et al., 2007). It has long been known that individual CesAs assemble into hexamer complexes that form the CSC rosette, but the number of CesAs in each lobe of the rosette has only recently been elucidated. It was thought that anywhere from 18-36 individual CesAs form a full CSC. As of 2014, the accepted model of the plant CSC is a hexamer of trimers assembled from 18 CesAs that produces 18 glucan chains per microfibril (Hill et al. 2014). The ratio of each CesA isoform in the complex is also a recent discovery, where both the PCW and SCW subunit stoichiometries are 1:1:1 in their respective complexes (Hill et al., 2014; Gonneau et al., 2014). Here, we contribute to the understanding of CesA stoichiometry by

analysis of CesA protein levels and stoichiometry across the developmental axis represented by the *Arabidopsis* inflorescence stem.

Chapter 2

Results

2.1 Purification and Specificity of CesA Antibodies

Previously, sensitive, isoform-specific antibodies to CesAs 1, 4, 7, and 8 were generated (Hill et al. 2014). However, to fully characterize CesA proteins in the *Arabidopsis* stem, isolation of highly specific antibodies to CesA3 and CesA6 was also required. Tien lab's existing Cesa3 and CesA6 antibodies each consisted of a mixture of antibodies that bind to different CesA-specific peptide epitopes (Table 1). To provide sufficient specificity, it was necessary to separate these antibody 'populations.' Peptide epitopes (Table 1) were individually conjugated to Sulfo-Link resin and epitope-specific antibodies were affinity purified from rabbit serum containing multiple populations of antibodies to CesA3 or CesA6. These epitope-specific antibodies were then tested for sensitivity and specificity by immunoblot, with anti-CesA3.2 and 6.1 chosen for all further immunoblot analysis (Fig. 1).

Table 1. Peptides used to generate CesA antibodies.

CesA 3.1	CKRLPYSSDVNQSPNRR
CesA 3.2	PQKEKISERMLGWHLTRGK
CesA 6.1	CGNNGIGFDQVSEGMSISRRNSGFPQSDLD
CesA 6.2	CRHEGDPDFEDGDDADF
CesA 6.3	CSISRRNSGFPQSDLD

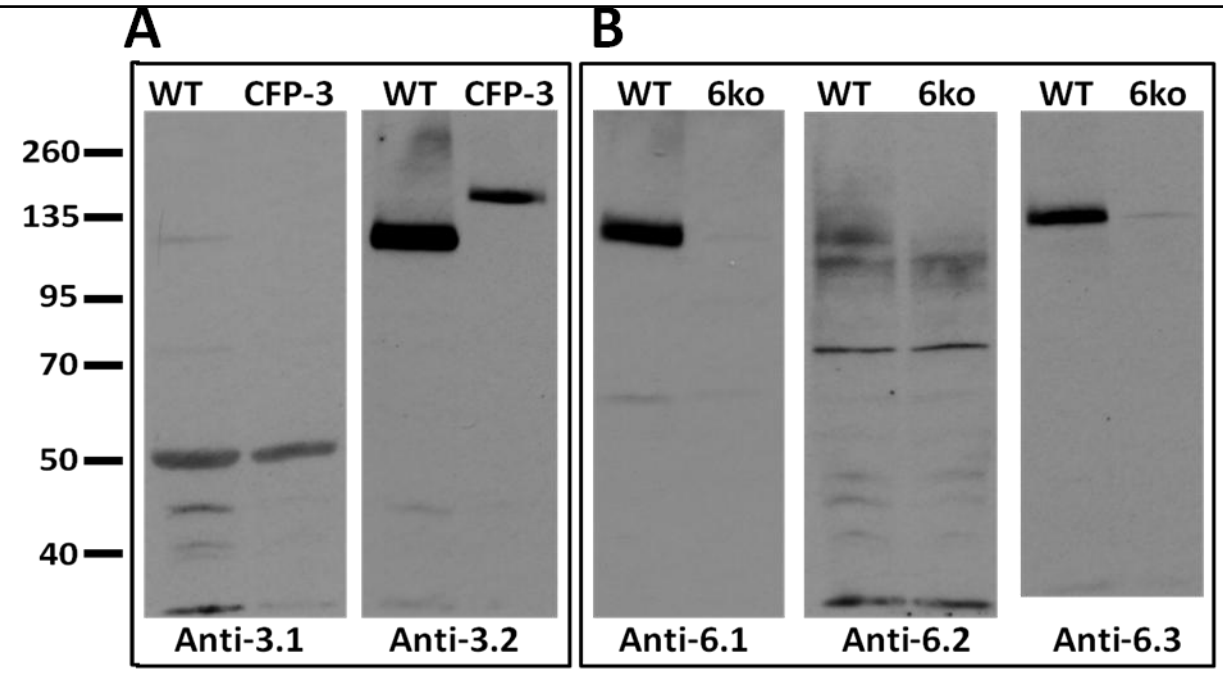


Figure 1. Purification and specificity of CesA antibodies by immunoblot.

Epitope-specific CesA purifications tested with seedling protein extracts to determine specificity and cross-reactivity. The CesA band is observed at 135 kDa. (A) anti-CesA3.1 fails to detect CesA3. Anti-CesA3.2 detects both CesA3 in WT samples and CFP-CesA3 in CFP-3 samples, with minimal other reaction. (B) Anti-CesA6.1 and 6.3 recognize CesA6 with minimal cross-reactivity in WT samples and little to no cross-reaction in *cesa6* knockout samples, while anti-CesA6.2 has no affinity to CesA6.

2.2 Quantification of CesA Protein Levels in the Arabidopsis Stem

The growing inflorescence stem of the model plant *Arabidopsis* represents a developmental gradient allowing for the study CesA expression during cell wall development. Wild-type stems were sectioned into 10% increments from 5-95% (Fig 2) and equal amounts (7.5 μg) of protein were separated by SDS-PAGE, transferred to nitrocellulose, and probed with

antibodies to CesAs 1, 3, 6, 4, 7, and 8, with the experiment being repeated in triplicate (Fig 3). Band intensity was measured by ImageJ and then a section was chosen and used to normalize the values within each immunoblot. Graphs represent the relative intensity of the normalized stem section immunoblots of their respective CesAs.

Levels of the primary cell wall CesAs (1, 3, and 6) show a close correlation along the stem, with maximal expression beginning 65% up the stem length measured from the bottom (Fig. 3A, B, C). A smaller PCW CesA expression peak is also observed at the base of the stem. Roughly equal CesA expression is observed along the rest of the stem.

Secondary cell wall CesAs (4, 7, and 8) show a similar patterning, but staggered back with respect to the primary cell wall CesAs (Fig. 4A, B). There is very low SCW CesA expression at the base and a gradual increase throughout the remainder of the stem length. Intensities corresponding to all three SCW CesAs increase sharply beyond 65% up the stem length.

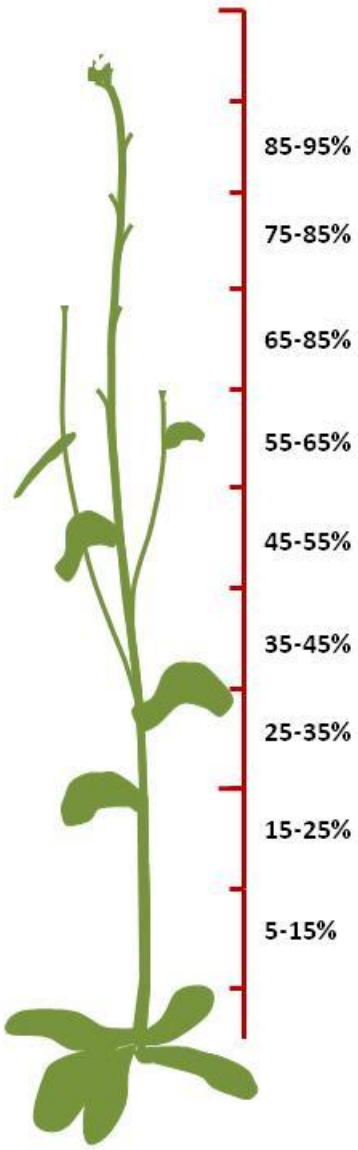


Figure 2. Arabidopsis model and sectioning scheme.

Once harvested, the stems are divided into ten percent increments for protein extraction. The top and bottom five percent are discarded.

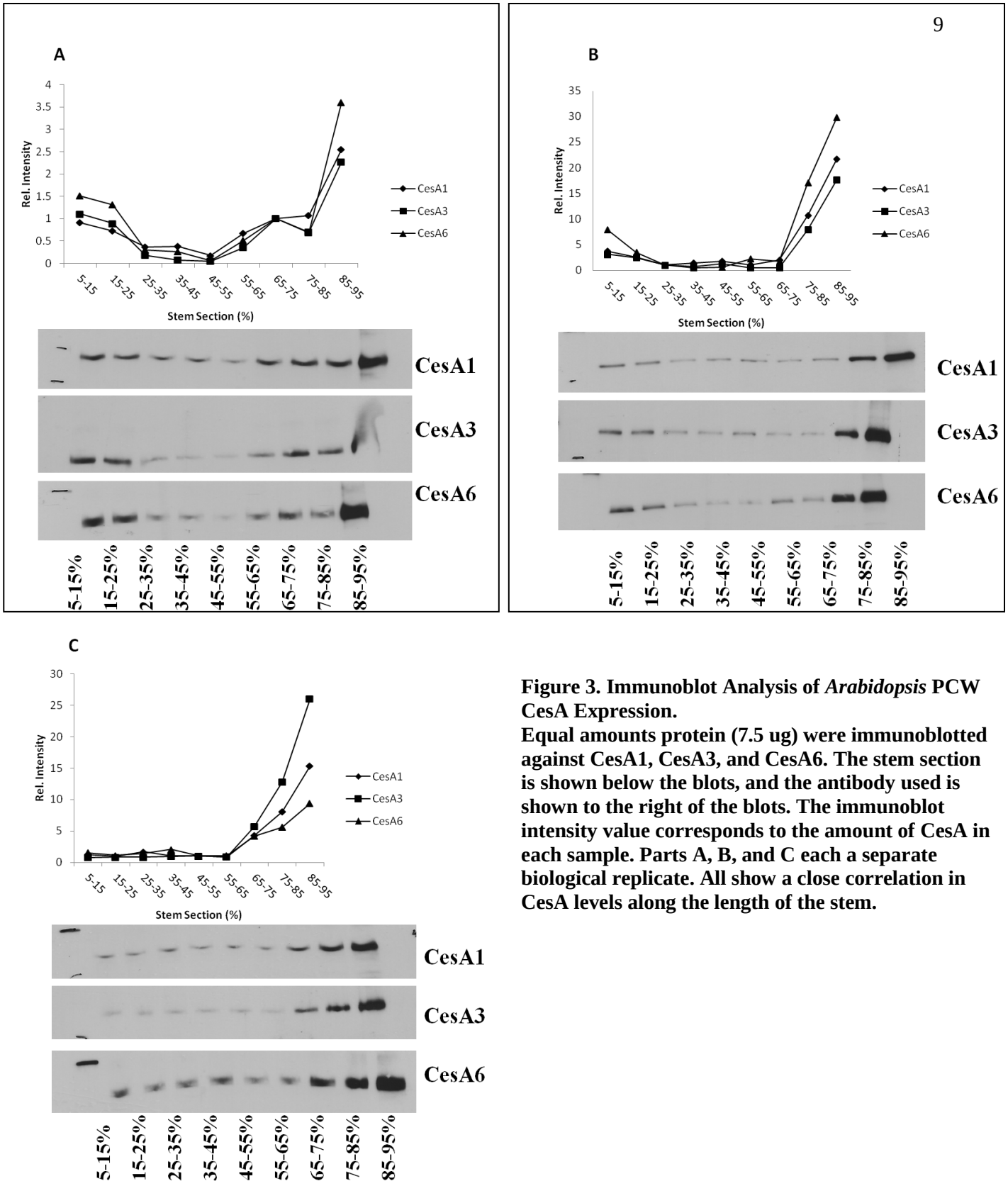
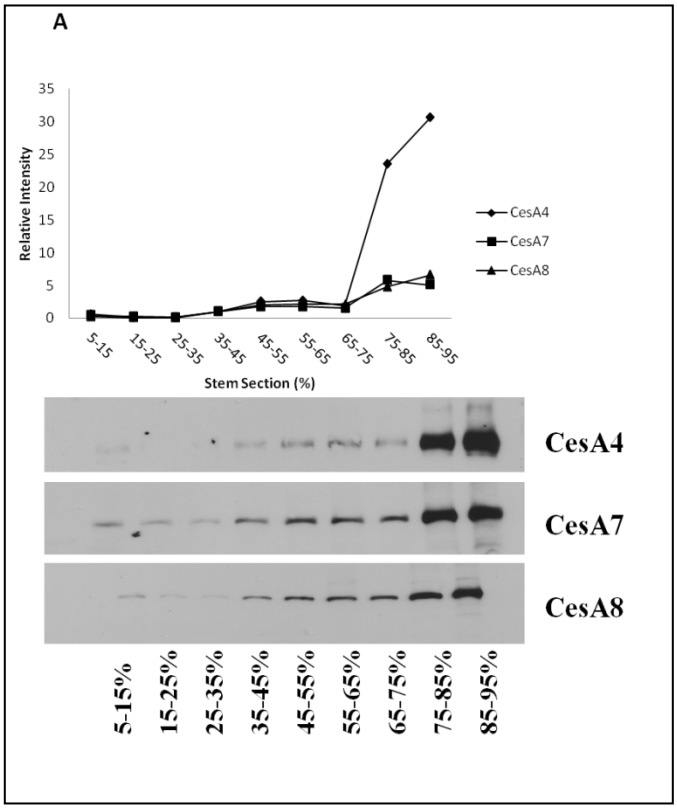


Figure 3. Immunoblot Analysis of *Arabidopsis* PCW CesA Expression.
Equal amounts protein (7.5 ug) were immunoblotted against CesA1, CesA3, and CesA6. The stem section is shown below the blots, and the antibody used is shown to the right of the blots. The immunoblot intensity value corresponds to the amount of CesA in each sample. Parts A, B, and C each a separate biological replicate. All show a close correlation in CesA levels along the length of the stem.



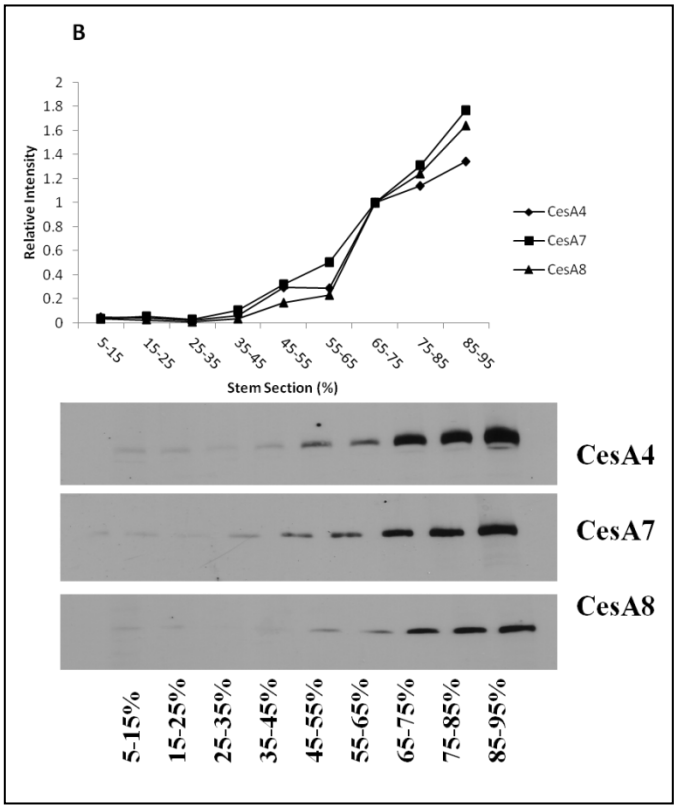


Figure 4. Immunoblot Analysis of *Arabidopsis* SCW CesA Expression.
Equal amounts protein (7.5 ug) were immunoblotted against CesA4, CesA7, and CesA8. The stem section is shown below the blots, and the antibody used is shown to the right of the blots. The immunoblot intensity value corresponds to the amount of CesA in each sample. Parts A and B each represent a separate biological replicate. All show a close correlation in CesA levels along the length of the stem.

Chapter 3

Discussion

Understanding of the CSC has progressed slowly since it was first visualized by freeze-fracture microscopy in 1976 (Brown et al., 1976). After decades, it was known to be a hexameric rosette structure, but the composition of each lobule remained elusive (Kimura et al., 1999). The *Arabidopsis* CSC is now known to be composed of ten CesA subunits depending on the type of wall synthesized, arranged in a certain stoichiometry in the lobes of the complex (Taylor et al., 2003; Gardiner et al., 2003; Persson et al., 2007). While many researchers investigated genetics or protein interactions in order to determine how these subunits are arranged, no clear picture has emerged as to how the CesA subunits assemble into the complete CSC.

Supported in part by this work, the Tien lab has contributed the knowledge of cellulose synthesis in higher plants by showing that CesA subunits specific to both primary and secondary cell walls assemble in a 1:1:1 stoichiometry (Hill et al., 2014). By mapping CesA expression in a developmental gradient across the *Arabidopsis* stem, this equimolar stoichiometry is shown to be fixed throughout development and provides support that no mixing of the primary and secondary cell wall CesAs occurs in the CSC.

3.1 Synthetic Peptides are Capable of Raising Antibodies specific to CesA isoforms

In previous coimmunoprecipitation and immunoblots analyses of CesA interactions, a portion of the *cesA* gene was expressed in bacteria and polyclonal antibodies were raised against

the purified gene products by a complicated, multistep purification process (Taylor et al., 2000, 2003; Desprez et al., 2007; Wang et al., 2008). Generally, they first amplified the genetic sequence corresponding to the n-terminus or variable region, inserted the PCR product into a plasmid vector, amplified the gene product in bacteria, and affinity purified the CesA peptide portion. This product was then injected into a rabbit or sheep to raise antibodies against the foreign protein, which were then purified from the serum.

In contrast to this complicated process, Hill Jr. et al. (2014) raised antibodies against chemically synthesized CesA peptide sequences unique to each isoform and injected them directly into a rabbit, eliminating the need for expression in a bacterial vector. Following the same procedure for this work, serum was purified after introduction of synthetic peptide sequences from CesAs 3 and 6 (Table 1). Antibodies purified by this method are highly specific and are not cross-reactive with other epitopes and non-CesA proteins (Fig. 1). Chemically synthesizing peptides is expensive, but the higher price is balanced by the speed of the process, hastened because there is no need to go through the lengthy process of expressing and purifying protein from bacteria. The CesA antibody synthesis presented here is a faster, easier alternative to producing high-affinity antibodies than previous procedures.

3.2 CesA Stoichiometry is Fixed Throughout Stem Development

Immunoblot analysis provides a quantitative analysis of CesA protein levels along the *Arabidopsis* inflorescence stem, which represents a spectrum of development that provides insight into the stoichiometry and assembly of the CSC. Primary and secondary cell wall CesA expression patterns can be clearly distinguished and explain where in the stem primary and

secondary cell wall cellulose are synthesized (Fig. 3, 4). PCW CesAs (1, 3, and 6) appear somewhat abundant at the very base of the stem, suggesting that some cellular process at the base requires cell expansion and growth, or at the least, primary cell wall cellulose. Meanwhile, very little expression is observed in the middle of the stem. Cells here are not depositing any primary cell wall. At about 65% up the stem, however, CesA expression increases dramatically, illustrating that cells at the stem tip are actively producing primary cell wall materials, as is expected – since the plant grows at the tip of the stem, the most new primary cell wall growth and thus CesA1/3/6 expression should be observed here.

Secondary cell wall CesAs (4, 7, and 8), however, show very low levels of expression at the base of the stem, providing evidence that these cells do not express SCW CesAs at this stage of development. In contrast to the sharp spike in PCW CesA expression, a gradual increase of SCW CesA expression occurs along much of the stem length. However, the highest amount of protein is seen at the tip of the stem, where it appears that both primary and secondary cell wall is actively synthesized.

Brown et al. (2005) have used coexpression of mRNA transcripts to identify what may occur during protein expression and implications for interactions. Real-time RT-PCR showed the CesA7 and 8 mRNA transcripts were most abundant at the base of the stem and decreased significantly up to the top of the stem (Brown et al., 2005). Their findings appear to contradict the expression pattern found in this work, but some important differences must be considered before comparing the results directly. First, Brown was measuring mRNA, not protein. Although levels of protein and RNA may be related, significant regulation takes place at this level, and so levels of each may not correlate. Second, the *Arabidopsis* analyzed by Brown was significantly younger than those used by this study. They reported the plants with 2-3

siliques on the stem, while our plants had 10+ siliques at the time of harvesting. These plants represented a different stage of development and perhaps a different mRNA and protein expression pattern. Their younger plants may have just begun laying down secondary cell wall at the base of the stem, explaining the high level of CesA7 and 8 expression. In contrast, by the eight week stage attained by our plants, it is possible that a secondary cell wall had already been deposited, in which case there is no reason for further SCW CesA expression at the base of the stem. Rather, secondary cell wall-expressing cells had stopped growing and secondary cell wall was being laid down higher up in the stem.

It is notable that both primary and secondary cell wall are being deposited at the top of the stem, and while no active secondary cell wall expression is observed at the base of the stem, primary cell wall presumably continues to be synthesized. This may suggest a mechanism of cell wall deposition by more than one type of cell for different purposes.

Tien labs' findings indicate an equimolar stoichiometry in both primary and secondary cell wall CesAs. The data presented in this work maps a developmental gradient of CesA expression supporting that this equimolar stoichiometry is fixed from the stem base to the tip. A fixed stoichiometry has implications for how the CSC is assembled, suggesting a fixed and defined pattern rather than random assembly, and providing an avenue for future investigation into the assembly of the CSC.

Distinct expression primary and secondary cell wall patterns are consistent with the theory that no mixing of subunits takes place between the PCW/SCW CSCs. While some research suggests that CesA1 can partly rescue a *cesa8ko* mutant, and CesA7 can substitute for CesA3, a different stoichiometric pattern would be expected if these were common replacements (Carroll et al., 2012; Li et al., 2013). The tight correlation of each cell wall protein pattern

discourages the idea of mixing, which would manifest as a decrease in expression of the redundant CesA, and an increase in expression of the CesA substituting for it. The observed fixed stoichiometry is a good explanation for the tight fit expression curves. Only at the uppermost part of the stem, where the most growth, and therefore protein expression, occurs, does this tight fit break down. The huge difference between band intensities from the stem tip and base is too high to be accurately captured by the film, which can only record about a 10x difference in intensity.

The findings on CesA stoichiometry presented here, along with the presumed organization of CesAs within the CSC presented by Hill Jr. et al. support a CSC model made from a hexamer of CesA trimers that synthesize an 18-strand cellulose microfibril, providing valuable new information on cellulose assembly and insight into the mechanism of cellulose synthesis.

Chapter 4

Materials and Methods

4.1 Plants and Growth Conditions

Arabidopsis thaliana plants were grown under 16-hour days at approximately 25°C in Pro Mix BX with Osmocote 10-10-10 fertilizer applied according to label. A thin layer of soil containing Dr. Pye's Scanmask Beneficial Nematodes was applied to cover the seeds to prevent fungus gnat larvae infestation. Plants were routinely culled to yield individuals growing at a similar rate. At approximately six weeks of age, for each experiment, 10 plants of similar height were harvested.

4.2 Affinity Purification of Peptide Antibodies

SulfoLink Coupling Gel was equilibrated to room temperature and 2 mL gel slurry was added to a chromatography column to give a 1 mL gel bed. The column was equilibrated with 4 volumes coupling buffer (50 mM Tris, 5 mM EDTA pH 8.5). To this column, 1 mg sulfhydryl-containing peptide, dissolved in 1 mL coupling buffer, was added to the column. The column was mixed for 15 minutes at RT and allowed to drain and 3 column volumes coupling buffer were used to wash the column. Unreacted groups on the SulfoLink resin were then blocked by the addition of 1 mL 50 mM L-cysteine HCL and mixed for 15 minutes. The column was washed with 6 column volumes wash solution (1 M NaCl), 2x 10 ml 1x PBS + 250 mM NaCl pH 7.2, 1x 10 ml Elution buffer (100 mM glycine pH 2.5), and 2x 10 ml 1x PBS pH 7.2. Total Ig protein (10 mL New Zealand white rabbit serum) was added to the column with 100 µL 10x PBS. The

column was then capped, wrapped with parafilm, and rotated overnight at 4°C. The next day, the column was equilibrated to room temperature rotating, and Ig was drained into a 15 mL tube,and labeled flow through. This flow through was then passed over the column once more at room temperature. The column was washed with 20 ml 1x PBS (pH 7.2) and 10 ml 1x PBS + 250 mM NaCl (pH 7.2). Antibody protein wad eluted 5 mL elution buffer (100 mM glycine pH 2.5), with 250-500 μL fractions taken into tubes containing 50 μL 1M Tris (pH 8) for immediate neutralization. A Bradford assay was performed to identify and pool peak antibody-containing fractions. Glycerol was added to 50% final concentration and antibodies were aliquoted and frozen at -80C.

4.3 Plant Stem Preparation and Phenol Protein Extraction

Ten plants of similar size were harvested at approximately 6 weeks of age. Branches, siliques, and leaves were removed to yield solely the primary inflorescence. Each stem was segmented in 10% increments as followed: 5-15%, 15-25%... 85-95%. These sections were snap frozen in liquid nitrogen, pooling the sections from the 10 plants (i.e., ten 5-15% sections pooled to make a single sample). Each sample was then ground by mortar and pestle with liquid N_2 into a fine powder. The powder was resuspended in acetone with 10% trichloroacetic acid and total protein was extracted (Wang et al., 2006). Final protein pellets were resuspended in 0.1xPBS + 1% SDS and protein concentration was determined by modified Lowry assay (Peterson, 1977). Protein samples were diluted to the same concentration in SDS-PAGE loading buffer before being subjected to SDS-PAGE and immunoblot analysis.

4.4 Western Blotting and Quantification

Equal amounts total stem protein from the extraction procedure above were run by 8% SDS-PAGE gel with 5% stacking gel (pH 8.8, 35% glycerol) and transferred to 0.1 μm-pore nitrocellulose (Whatman), which were then dried. Blots were blocked in Tris-buffered saline (TBS, 0.5% Tween 20), and washes, primary antibody, and secondary antibody were diluted in TBS. Blots were developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo) and exposed on CL-Xposure Film (Thermo). Digitized blot band intensities were quantified by ImageJ software (National Institutes of Health). Relative intensity of the normalized stem section immunoblots correlate to relative protein in each stem section.

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